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Breast Cancer Therapy

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13. ABSTRACT (Maximum 200 Words) Matrix Metalloproteinases (MMPs), a family of over 20 types of enzymes, collectively are capable of degrading all the components of the extracellular matrix. MMP-2 and MMP-9 (also known as gelatinases) are specifically thought to play critical roles in tumor cell invasion and are frequently co-expressed in breast cancer. Cyclic peptides containing the sequence HWGF have been described as selective inhibitors of MMP-2 and MMP-9. We tested the hypothesis that gelatinase expression may provide a target for in vivo tumor imaging using a radiolabeled gelatinase inhibitor. The peptide, DOTA-CTTHWGFTLC (DOTA-CTT), was labeled with Cu-64 [$T_{1/2} = 12.7$ h], which has a decay scheme suitable for both PET imaging and cancer therapy. This conjugate maintained MMP-2 inhibitory activity comparable to Ilomastat, a broad-range inhibitor of MMPs. An increase in the MMP-2/9 activity of human metastatic breast cancer MDA-MB-435 tumors in nude mice was observed from 4 to 10 weeks post-implantation. MicroPET images of Cu-64-DOTA-CTT in the tumor-bearing nude mice showed tumor uptake at 8-wk post-implantation; however, the same mouse with 5-wk palpable tumors showed no uptake of the tracer, suggesting that the MMP-2 and MMP-9 activity is related to the stage of tumor growth. These data suggest the potential of radiolabeled gelatinase inhibitors as markers for imaging the metastatic capability of human breast cancer.				
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Introduction

Matrix Metalloproteinases (MMPs) are a family of at least 15 types of enzymes, both secreted and membrane-bound zinc endopeptidases. Collectively, they are capable of degrading all the components of the extracellular matrix. Studies suggest that MMPs are utilized in cancer, and facilitate both local tumor invasion and metastasis. MMP-2 and MMP-9 (also known as gelatinases), in particular, are thought to play critical roles in tumor cell invasion and are frequently co-expressed in human cancers. The gelatinases are cell secreted enzymes and have been implicated in the progression of breast cancer. Moreover, it has been shown that MMP inhibitors can also inhibit angiogenesis.

The goal of the project was to test a hypothesis regarding the ability to image by positron emission tomography (PET) the expression of MMP-2 and MMP-9 in a metastatic tumor model using radiolabeled MMP-2 and MMP-9 peptide inhibitors. The hypothesis to be tested was that the accumulation of a radiolabeled gelatinase inhibitor will correlate with gelatinase enzyme activity determined in the tumor in an *ex vivo* assay. It has been demonstrated by Koivunen et al. that cyclic peptides containing the sequence HWGF are selective inhibitors of MMP-2 and MMP-9 (1). The synthetic cyclic peptide, CTTHWGFTLC, suppressed migration of breast cancer tumor cells in vitro and prevented the growth and invasion of MDA-MB-435 human breast cancer tumors in mice, suggesting that this peptide has potential as an anticancer agent. These data by Koivunen et al. strongly suggest that this peptide localizes to gelatinase, both in cell culture and *in vivo*. We have demonstrated the effectiveness of ^{64}Cu ($T_{1/2} = 12.7$ h) as a radionuclide for PET imaging with other tumor receptor binding peptides in both animal models and humans. For this project, we conjugated CTTHWGFTLC with the chelator DOTA (1,4,7,10-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid) for labeling with ^{64}Cu . Presented in this report are results on the inhibitory activity of DOTA-CTT, the gelatinase expression in a human breast cancer tumor-bearing mouse model, and *in vivo* imaging of ^{64}Cu -DOTA-CTT in the tumor-bearing mouse model.

Body

In the approved Statement of Work, we proposed the following specific aims:

- 1) To derivatize CTTHWGFTLC at the N-terminus with the chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for radiolabeling with the positron-emitting and/or therapeutic radionuclides Y-90 ($T_{1/2} = 64$ h; 100% beta minus), Y-86 ($T_{1/2} = 15$ h; 33% positron) and Cu-64 ($T_{1/2} = 12.7$ h; 40% beta minus, 17.6% positron)
- 2) To demonstrate that these derivatized peptides maintain their MMP-2 and MMP-9 inhibitory activity utilizing commercially available assay kits
- 3) To compare the biodistributions in MDA-MB-435 human breast cancer tumor-bearing mice of the Y-86/90- and Cu-64-DOTA-CTTHWGFTLC analogs with analogous radiolabeled control, non-specific peptides
- 4) To perform therapy studies with the Y-90 and Cu-64 -labeled DOTA-CTTHWGFTLC in MDA-MB-435 tumor-bearing

Thus far we have accomplished progress towards Aims 1-3 using only one radionuclide, ^{64}Cu . The progress towards these aims is described in detail below.

AIM 1) SYNTHESIS AND RADIOLABELING OF ^{64}Cu -DOTA-CTTHWGFTLC (^{64}Cu -DOTA-CTT)

CTTHWGFTLC was synthesized by solid-phase methods, and then derivatized at the N-terminus with DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) as previously described (2). Radiolabeling with ^{64}Cu was carried out by addition of 1-5 mCi (37-185 MBq) of ^{64}Cu in 0.1 M ammonium acetate (pH 6.5) to 1-5 μg of the peptide in 0.1 M ammonium acetate followed by 30 minutes incubation at

room temperature as previously reported (3). The ^{64}Cu -labeled DOTA-CTTHWGFTLC (^{64}Cu -DOTA-CTT) was purified on a C-18 SepPak Light cartridge, using 100% ethanol as the elution solvent, and radiochemical purity was determined by radio-TLC or radio-HPLC.

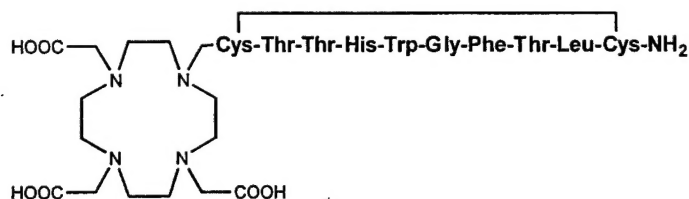


Figure 1: Structure of DOTA-CTT

AIM 2) DETERMINATION OF THE RELATIVE INHIBITORY ACTIVITY OF DOTA-CTT

Gelatinase activity assays for MMP inhibitor peptides was developed during the funding period. Gelatinase activity with increasing concentrations of inhibitor was determined in the linear range using a biotinylated gelatinase substrate cleavage assay kit (Chemicon, Temecula, CA). Gelatin, denatured collagen type I, is the most common and inexpensive protein substrate that is highly susceptible to cleavage by MMP-2 and MMP-9, while being less efficiently cleaved by many other MMPs. To determine the inhibitory activity of DOTA-CTT, activated MMP-2 (20 pM) was preincubated for 30 min with DOTA-CTT at increasing concentrations (0.01 – 150 nM) in 96-well plates. The inhibitory activity of the DOTA-peptide was compared to Ilomastat (Chemicon, Temecula, CA), a broad-range hydroxamate inhibitor of MMPs. Curve fitting and data analysis were performed using Prism software (Graph Pad, San Diego, CA).

Figure 2 shows the inhibitory curve of MMP-2 activity with DOTA-CTT and Ilomastat. Increasing concentrations of DOTA-CTT and Ilomastat efficiently inhibited MMP-2. DOTA-CTT showed comparable inhibitory activity to MMP-2 as Ilomastat.

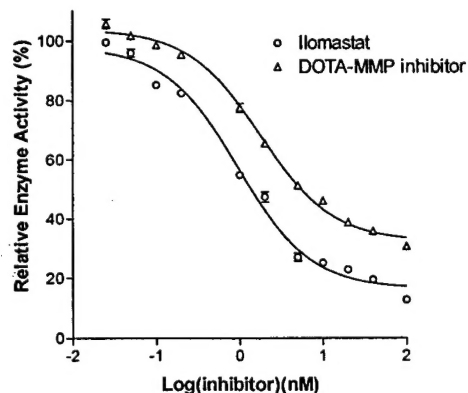


Figure 2: MMP-2 inhibition with DOTA-CTT and Ilomastat. Increasing concentrations of DOTA-CTT and Ilomastat were added to 20 pM APMA-activated MMP-2 in a 30 min incubation. Each data point represents the mean value of triplicate measurements. The gelatinolytic activity of 20 pM APMA-activated MMP-2 is assumed to be 100%. The IC_{50} of Ilomastat is 0.95 nM (0.79-1.15 nM, 95% CI), while the IC_{50} of DOTA-CTT is 1.78 nM (1.58-2.0 nM, 95% CI).

DETERMINATION OF THE MMP-2 ACTIVITY IN MDA-MB-435 TUMOR SAMPLES

Tumor extracts from MDA-MB-435 human breast cancer tumor-bearing mice were obtained as described (4). Fragments of tumor tissue were suspended in 0.1 M Tris-HCl buffer, pH 7.8, with 0.15 M NaCl, 10 mM CaCl_2 and 0.02% sodium azide (TNC buffer) and homogenized in a Tissumizer (Tekmar Company, Cincinnati, OH). Homogenates were centrifuged at 5,000 g for 30 min at 4° C, and supernatants were saved for the gelatinase activity assay. Protein determination was done in supernatant samples by using BCA protein assay kit (Pierce, Rockford, IL). The samples were analyzed immediately or stored at -80° C.

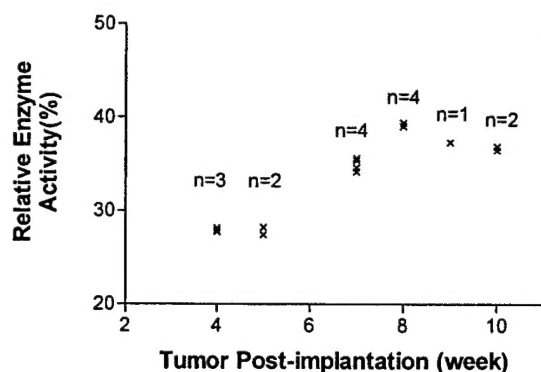


Figure 3: Relative gelatinase activity of MDA-MB-435 tumor supernatant samples (10 μ g protein) at various times post-implantation in female nude mice. The gelatinase activity of 10 ng activated MMP-2 was used as a reference (100%). The n values signify the number of tumors evaluated per week. The values are a mixture of subcutaneous (s.c.) and mammary fat pad tumor implantations.

Gelatinase activity of the tumor supernatant samples was determined in the linear range using the biotinylated gelatinase substrate cleavage assay kit (Chemicon, Temecula, CA). Purified proenzyme MMP-2 (Oncogene Research Products, Cambridge, MA) was used as the positive control. Gelatinase activity was detected in tumor tissue extracts from 4 weeks tumor post-implantation through 10 weeks (Figure 3), showing an increase on weeks 7-10 compared to weeks 4 and 5.

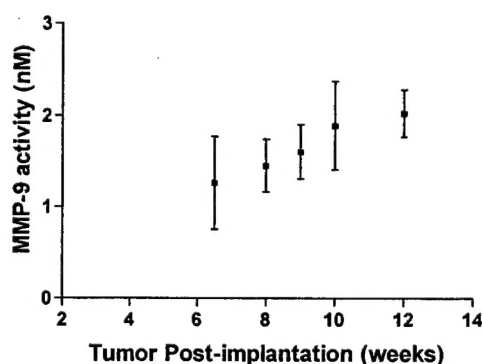


Figure 4. MMP-9 activity in MDA-MB-435 tumors in nude mice from 7 to 12 weeks post-implantation using a fluorogenic substrate assay.

A fluorogenic peptide substrate was applied to determine MMP-9 activity of tumor extracts in the linear range. MMP-9 activity in MDA-MB-435 tumors grown in nude mice was determined using DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH₂ (Calbiochem, San Diego, California). The substrate was prepared as a 5 mM stock solutions in dimethyl sulfoxide. Fluorescent assays were measured at $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 465$ nm using a microtiter plate reader. MMP-9 (Calbiochem, San Diego, California) were used as a standard in the experiment. Activation of MMP-9 was performed by incubating the enzyme in 0.5 mM 4-aminophenyl mercuric acetate (APMA) (Sigma, St. Louis, MO) (borate solution (0.05 M, pH 9.0)) for 2 hr at 37° C. A typical assay was carried out by incubating substrate (10 nM final concentration) and activated MMP-9 (0-10 nM) or tumor supernatant samples (30 μ g) for 40-60 minutes in a black 96-well plate. The amount of substrate hydrolysis was calculated based on the fluorescence values of substrate solution after subtraction of the reaction blank value.

AIM 3) MICROPET IMAGING OF ⁶⁴Cu-DOTA-CTT IN TUMOR-BEARING MICE

Positron emission tomography (PET) imaging was performed on the first commercially available microPET® (Concorde Microsystems, Knoxville, TN), which was based on the design of Cherry and colleagues (5). ⁶⁴Cu-DOTA-CTT was prepared using previously described methods (2) in $\geq 98\%$ radiochemical purity. Specific activity was 1500-2000 Ci/mmol (56 to 63 GBq/mmol). Imaging studies were carried out on three female nude mice carrying MDA-MB-435 tumors. Two mice bearing tumors in the mammary fat pad were imaged on the microPET® 5 weeks after tumor implantation. Mice were injected with 300-350 μ Ci ⁶⁴Cu-DOTA-CTT (~ 0.2 μ g) and imaged at 1, 2, 4, and 24 h post-injection. The two mice were imaged side by side and remained in the same bed position for all time points. One of two mice imaged at 5 weeks tumor post-

implantation was imaged again after 8 weeks post-implantation with another mouse following the same imaging protocol.

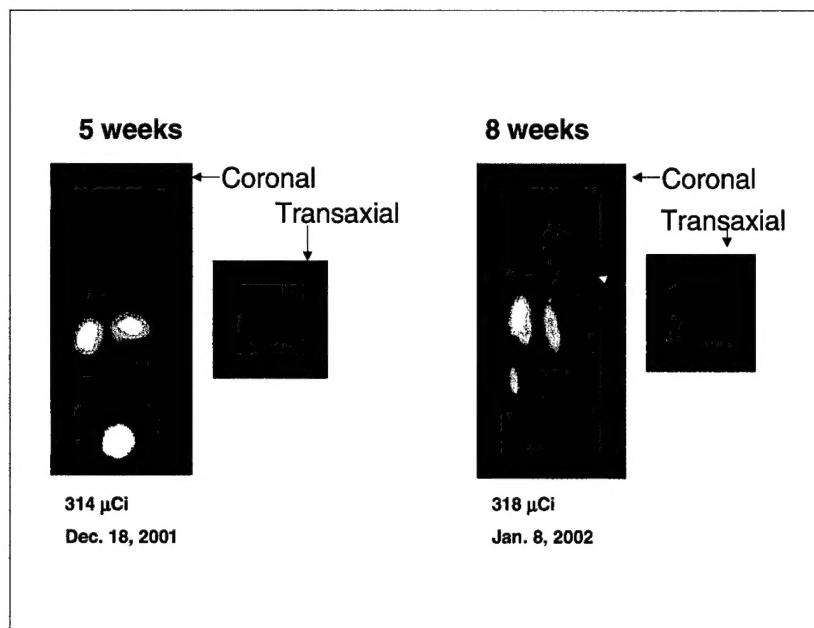


Figure 5: MicroPET images of ^{64}Cu -DOTA-CTT 1 hr post-injection in MDA-MB-435 tumor bearing female nude mouse after 5 weeks (left) and 8 weeks (right) tumor post-implantation.

Figure 5 shows the coronal and transaxial microPET® images of a MDA-MB-435 tumor-bearing nude mouse, 1 h post-administration of ^{64}Cu -DOTA-CTT, at 5 and 8 weeks tumor post-implantation. Uptake in the mammary fat pad tumor was not visible after 5 weeks tumor post-implantation at all post-injection times examined. Conversely, the same mouse showed significant uptake in tumor at 8 weeks tumor post-implantation at 1 and 2 h post-injection, while wash-out of the ^{64}Cu activity became apparent in the 4 h post-injection images. The ^{64}Cu time-activity curves in the tumor, kidney and liver were generated by measuring regions of interest (ROIs) that encompassed the entire organ from the microPET® images in MDA-MB-435 tumor bearing nude mice (Figure 6).

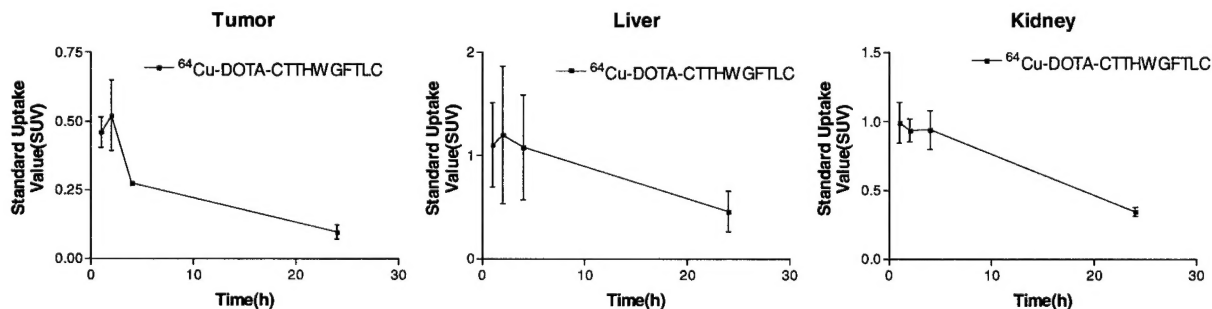


Figure 6: Time activity curve generated by drawing ROIs over the liver and kidney from ^{64}Cu -DOTA-CTTHWGFTLC microPET of MDA-MB-435 tumor bearing nude mice ($n = 2$).

Key Research Accomplishments

The major accomplishments of this project were:

- the synthesis and ^{64}Cu -labeling of a chelator-peptide conjugate, DOTA-CTT
- the determination of the MMP-2 inhibitory activity in a biological assay

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- the validation of a breast cancer tumor-bearing nude mouse model that expresses both MMP-2 and MMP-9
- the demonstration that the gelatinase activity levels in the MDA-MD-435 tumor grown in nude mice increases with time post-implantation
- progress towards the proof-of-principle that gelatinase expression can be imaged *in vivo*.

Reportable Outcomes

The research funded by DoD has been or will be presented at three meetings. We anticipate a manuscript will be written and submitted for publication in the next 6 months.

1. Li WP, Lewis JS, Lan S, Anderson CJ. ^{64}Cu -DOTA-CTTHWGFTLC, a selective gelatinase inhibitor for tumor imaging. *Journal of Nuclear Medicine* 2002; 43:277P.
2. Anderson CJ, Li WP, Lewis JS, Eiblmaier M, Lan S. ^{64}Cu -DOTA-CTTHWGFTLC, a radiolabeled gelatinase inhibitor for PET imaging of metastatic breast cancer. Oral Presentation, Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, Orlando, FL September, 2002.
3. Li WP, Lewis JS, Eiblmaier M, Lan S, Anderson CJ. In vitro and in vivo evaluation of a radiolabeled gelatinase inhibitor for microPET imaging of metastatic breast cancer. Accepted for Oral Presentation, Academy of Molecular Imaging Annual Meeting, San Diego, CA, October, 2002.

Grant submitted to NIH: Targeting Matrix Metalloproteinases for Tumor Imaging; Carolyn J. Anderson, P.I., \$125,000/year for 2 years.

This grant was reviewed in June, 2002 and was not given a fundable score. A revised grant application will be submitted in October, 2002.

Conclusions

The research carried out under this funding defined a new class of cancer imaging agent based on matrix metalloproteinase (gelatinase) expression. The data generated under this funding suggest that gelatinase levels in a breast cancer tumor implanted in mice, increase with the time post-implantation. If continued progress is made, future funding to develop imaging agents that may be worthy of clinical evaluation. The implications of this are that if in a human situation, gelatinase levels of certain tumor types, such as breast cancer, may change with time. An imaging agent that can somehow quantify gelatinase expression may provide information on the metastatic potential of the patient's tumor. Interventions, such as specific types of cancer therapeutic drugs, could be performed in advance of actual metastasis, possibly increasing survival of the cancer patient.

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Appendices

A copy of the abstracts listed below is included as appendix material.

1. Li WP, Lewis JS, Lan S, Anderson CJ. ^{64}Cu -DOTA-CTTHWGFTLC, a selective gelatinase inhibitor for tumor imaging. *Journal of Nuclear Medicine* 2002; 43:277P.
2. Anderson CJ, Li WP, Lewis JS, Eiblmaier M, Lan S. ^{64}Cu -DOTA-CTTHWGFTLC, a radiolabeled gelatinase inhibitor for PET imaging of metastatic breast cancer. Oral Presentation, Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, Orlando, FL September, 2002.
3. Li WP, Lewis JS, Eiblmaier M, Lan S, Anderson CJ. In vitro and in vivo evaluation of a radiolabeled gelatinase inhibitor for microPET imaging of metastatic breast cancer. Accepted for Oral Presentation, Academy of Molecular Imaging Annual Meeting, San Diego, CA, October, 2002.

⁶⁴Cu-DOTA-CTTHWGFTLC, A SELECTIVE GELATINASE INHIBITOR FOR TUMOR IMAGING. W. P. Li*, J. S. Lewis, S. Lan, C. J. Anderson, Washington University School of Medicine, Saint Louis, MO. (201203)

Objectives: The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases involved in the degradation of the extracellular matrix. MMPs have been implicated in the process of tumor growth, invasion, and metastasis, and their overexpression in various types of tumors is often correlated with an adverse prognosis in patients with cancer. Two MMPs closely associated with metastatic potential are 72 kDa MMP-2 (gelatinase A) and 92 kDa MMP-9 (gelatinase B). Metastatic tumor cell lines, including lung, colon, breast, and pancreatic carcinomas, are known to express higher levels of gelatinases than their nonmetastatic counterparts. Cyclic peptides containing the sequence HWGF have been described as selective inhibitors of MMP-2 and MMP-9 (Koivunen et al., *Nature Biotech* 17;1999:768-774). We propose that MMP expression may provide a target for in vivo tumor imaging using a radiolabeled MMP inhibitor. **Methods:** The peptide CTTHWGFTLC was synthesized by

solid-phase methods, conjugated at the N-terminus with DOTA (1,4,7,10-tetraazacyclododecane- N,N',N'',N'''-tetraacetic acid) and labeled with ⁶⁴Cu (T_{1/2} = 12.7 h; 39% β⁻; 17.4 β⁺). **Results:** The inhibitory activity of this conjugate was determined in a gelatinase activity assay, and the results demonstrated that DOTA-CTTHWGFTLC maintained MMP-2 and MMP-9 inhibitory activity comparable to Ilomastat, a broad-range hydroxamate inhibitor of MMPs. Furthermore, an increase in the gelatinolytic activity of MMPs in human breast cancer MDA-MB-435 tumor-bearing nude mice was observed from 5 to 7 weeks post-implantation. Preliminary microPET (Concorde Microsystems) images of ⁶⁴Cu-DOTA-CTTHWGFTLC in MDA-MB-435 tumor bearing nude mice showed significant tumor uptake in mice with larger tumors, but not in mice with smaller tumors, suggesting that the MMP-2 and MMP-9 activity is possibly related to the stage of tumor growth. **Conclusion:** These data suggest the potential of radiolabeled MMP inhibitors as markers of cancer metastasis.

CU-64-DOTA-CTTHWGFTLC: A RADIOLABELED GELATINASE INHIBITOR FOR PET IMAGING OF METASTATIC BREAST CANCER

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Matrix Metalloproteinases (MMPs) are a family of over 20 types of enzymes, both secreted and membrane-bound zinc endopeptidases, which collectively are capable of degrading all the components of the extracellular matrix. Studies suggest that MMPs are utilized in cancer, and facilitate both local tumor invasion and metastasis. MMP-2 and MMP-9 (also known as gelatinases) are specifically thought to play critical roles in tumor cell invasion and are frequently co-expressed in human cancers, in particular, breast cancer. Cyclic peptides containing the sequence HWGF have been described as selective inhibitors of MMP-2 and MMP-9. We tested the hypothesis that gelatinase expression may provide a target for in vivo tumor imaging using a radiolabeled gelatinase inhibitor. The peptide, CTTHWGFTLC, was synthesized by solid-phase methods, conjugated at the N-terminus with the chelator DOTA (1,4,7,10-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid) and labeled with Cu-64 [$T_{1/2} = 12.7$ h; 39% beta minus; 17.4% positron], a radionuclide with a decay scheme suitable for both imaging by positron emission tomography (PET) and cancer therapy. The inhibitory activity of this conjugate was determined in a gelatinase activity assay and the results demonstrated that DOTA-CTTHWGFTLC maintained MMP-2 inhibitory activity comparable to Ilomastat, a broad-range hydroxamate inhibitor of MMPs (IC₅₀'s of 1.78 nM vs 1.15 nM, respectively). Zymography of excised MDA-MB-435 tumor from nude mice showed the presence of MMP-2 and MMP-9. A relative increase in the gelatinolytic activity of human metastatic breast cancer MDA-MB-435 tumors grown in nude mice was observed from 5 to 8 weeks post-implantation. Preliminary microPET images of Cu-64-DOTA-CTTHWGFTLC in MDA-MB-435 tumor-bearing nude mice showed significant tumor uptake in mice at 8-weeks post-implantation; however, the same mice with 5-week old palpable tumors showed no observable uptake of the tracer, suggesting that the MMP-2 and MMP-9 activity is possibly related to the stage of tumor growth. These data suggest the potential of radiolabeled gelatinase inhibitors as markers for imaging the metastatic capability of human breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0666 supported this work.

Li WP, Lewis JS, Eiblmaier M, Lan S, Anderson CJ. In vitro and in vivo evaluation of a radiolabeled gelatinase inhibitor for microPET imaging of metastatic breast cancer. Accepted for Oral Presentation, Academy of Molecular Imaging Annual Meeting, San Diego, CA, October, 2002.

IN VITRO AND IN VIVO EVALUATION OF A RADIOLABELED GELATINASE INHIBITOR FOR MICROPET IMAGING OF METASTATIC BREAST CANCER

W. P. Li, J. S. Lewis, M. Eiblmaier, S. Lan, C. J. Anderson

Siteman Cancer Center and The Mallinckrodt Institute of Radiology, Washington University School of Medicine

Matrix Metalloproteinases (MMPs) are enzymes responsible for the breakdown of proteins of connective tissue. MMPs can regulate the tumor microenvironment, and their expression is increased in most cancers compared with normal tissue. MMP-2 and MMP-9 (gelatinases) are most closely correlated with metastatic potential in human cancers. Cyclic peptides containing HWGF have been described as selective gelatinase inhibitors. We hypothesized that gelatinase expression may provide a target for imaging tumor metastatic potential using a radiolabeled gelatinase inhibitor. The CTTHWGF₁₋₁₈ (CTT) peptide, a selective inhibitor of gelatinases, was conjugated to DOTA for radiolabeling with Cu-64 for microPET imaging in metastatic tumor-bearing mice. A gelatinase inhibitory assay demonstrated that DOTA-CTT maintained MMP-2 inhibitory activity comparable to Ilomastat, a broad-range MMPs inhibitor. Zymography of excised MDA-MB-435 tumors from mice confirmed the presence of MMP-2 and MMP-9. A consistent increase of gelatinolytic activity in tumor extracts was observed from 6 to 12 weeks post-implantation by a fluorogenic assay. In vivo activity biodistribution of Cu-64-DOTA-CTT in MDA-MB-435 tumor-bearing mice at stages of disease was monitored in the high resolution microPET. Preliminary microPET images showed significant tumor uptake at 8-weeks post-implantation; however, the same mice with 5-week old palpable tumors showed no visible uptake, suggesting that the gelatinolytic activity is possibly related to the stage of tumor growth. MicroPET has delineated gelatinolytic activity without the need of necropsy and could be used to screen libraries of peptide. These data suggest the potential of radiolabeled HWGF peptides for imaging the metastatic capability of human breast cancer.

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Basic Science Categories. Small Animal Imaging
Young Investigator Category